NITROGEN AVAILABILITY AS AN AID IN THE DIFFERENTIATION OF BACTERIA IN THE COLI-AEROGENES GROUP

N. B. MITCHELL AND MAX LEVINE

Laboratory of Food and Sanitary Bacteriology, Iowa State College, Ames, Iowa

The colon group of bacteria may be characterized as non-sporing Gram-negative bacilli which ferment lactose, producing acid and gas, and which can grow aerobically. Although many bacteriologists recognize the genera *Escherichia* and *Aerobacter*, the genus *Citrobacter* has not been generally accepted.

In 1923, Koser showed that strains of the genus *Aerobacter* utilized citric acid as a sole source of carbon (citrate positive), whereas those of the genus *Escherichia* did not (citrate negative). In 1924, Koser found that 23 of 25 coliform strains [methyl-red (+), Voges-Proskauer (−)] isolated from unpolluted soil were citrate (+). Due to their source he referred to them as "soil forms." The possible sanitary significance of this finding was immediately realized. The correlation of habitat with morphology and biochemical characteristics was, and still is, the chief hope for an improved criterion of water pollution.

Bardsley (1934) summarized previous work on this point: "Most workers in tropical countries where pollution is very heavy find it practical to distinguish between the different types of coliform bacilli, and only those organisms which conform to the true *B. coli* group [methyl-red (+), citrate (−)], are regarded as excretal contamination . . . . In temperate climates the position is not so well defined."

Fermentation of cellobiose and alpha-methyl-glucoside, production of trimethylene glycol from glycerol, and even Werkman and Gillen's (1932) description of the genus *Citrobacter* as comprising those citrate (+) coli-like strains which produce "Ace-

toin . . . rarely from glucose and then only in traces" have been found inadequate for differentiation of the "intermediates" from strains of the genera *Escherichia* and *Aerobacter*. A clearer characterization of the "intermediate" group is necessary.

The physiological characterization of the colon group has been stressed, in the past, from the standpoint of carbohydrate chemistry though some work with several purine compounds has been reported. Plenge (1903) and Schittenhelm and Schröter (1903, 1904) reported that "*B. coli*" attacked nucleic acid. Koser (1918) showed that *Aerobacter*, but not *Escherichia* strains, utilized uric acid as a sole nitrogen source. He obtained similar results with hypoxanthine hydrochloride, although here the *Aerobacter* strains grew less luxuriantly. Chenn and Rettger (1920) confirmed Koser's work on uric acid and found that xanthine gave similar results with typical *Escherichia coli* strains. But of 20 coli-like strains from soil, 10 of which were uric acid (+) and 10 uric acid (−), all were xanthine (−).

Nucleic acid and its degradation products were selected for this study because (1) of the known fact that uric acid is an available nitrogen source for *Aerobacter* but not for *Escherichia* strains; and, (2) of the possibility of correlating the findings with work in biochemistry.

According to Levene (1920), yeast nucleic acid is composed of the following radicals: 4 phosphate, 4 carbohydrate, 2 purine (adenine and guanine), and 2 pyrimidine (uracil and cytosine). Uric acid is the main end-product of purine digestion in man and anthropoid apes; allantoin is the chief end-product in most other animals. Allantoin is converted, *in vitro*, to hydantoin and urea, and further to glycine, carbon dioxide and ammonia.

The following investigation of the availability of yeast nucleic acid and some of its degradation products, (adenine sulphate, xanthine, uracil, uric acid, allantoin, hydantoin, and urea) was made with a view to development of differential media for the colon group. All but urea were Eastman products.

The basal medium consisted of: 0.5 per cent NaCl; 0.02 per cent MgSO$_4$; 0.004 per cent brom-thymol blue; and approximately 2 per cent by volume of 1.0 molar phosphate buffer

(pH 7.1). In some media it was necessary to employ a more alkaline phosphate buffer solution in order to adjust the reaction to approximately pH 7.1. To determine the availability of a compound as a source of nitrogen, 0.2 per cent of glucose and 0.05 per cent of the test compound were added to the basal medium; to ascertain the availability of a compound as a sole source of carbon, 0.2 per cent of monoammonium phosphate was supplied as an available nitrogen source, and to determine the availability of a compound as a sole source of both carbon and nitrogen, 0.5 per cent of the test compound was added to the basal medium described above.

All glassware was acid washed and rinsed with sodium bicarbonate solution followed by distilled water. All chemicals were chemically pure. The constituents were dissolved in conductivity water at a temperature below boiling. Since the effect of autoclaving these compounds was unknown, all media were sterilized by filtration through Chamberland L3 candles. Inoculums consisted of a one-millimeter loop of a 24-hour broth culture. Incubation was for four to five days at 30°C. (temperature of the medium).

The bacteria employed in the nitrogen availability studies included: 106 *Escherichia*; 39 *Aerobacter cloacae*; 31 *Aerobacter aerogenes* (indol positive); 41 *Aerobacter aerogenes* (indol negative); 138 "intermediate" strains (75 of which were contributed by workers outside this laboratory); and 6 strains which were received as "intermediates" but which gave questionable Voges-Proskauer reactions. Included among the "intermediate" strains was a transfer of the original strain of *Citrobacter freundii*, the type species of the genus *Citrobacter*.

Frozen eggs constituted the source of 90 per cent of *Escherichia*, 33 per cent of "intermediate," 77 per cent of *Aerobacter*, and one of the 6 Voges-Proskauer questionable strains. The source of isolation was unobtainable for 48 per cent of the "intermediate" and 3 of the Voges-Proskauer questionable strains. The rest of the cultures were secured from fowl or human feces, surface water, swimming pool water, etc. The date of isolation, or of arrival at this laboratory, varied from 1929 to 1936. The "in-

termediate" and questionable Voges-Proskauer strains were purified by at least three platings on eosin methylene-blue agar. The *Escherichia* and *Aerobacter* strains, from the Iowa State College bacteriology laboratory collection, were checked, as to lactose, eosine methylene-blue agar, Voges-Proskauer, and citrate reactions.

TABLE 1

Summary of differential characteristics of the coli-aerogenes group

	ESCHE-RICHIA	INTER-MEDIATE	AERO-BACTER	VP (?)	CITROBACTER FREUNDII
	Number of strains				
	106	138	111	6	1
	Per cent of positive reactions				
Yeast nucleic acid........	0	0	96	100	—
Uric acid.................	0	0	100	100	—
Allantoin.................	0	0	100	100	—
Hydantoin...............	0	0	87	100	—
Uracil...................	80	2	100	100	—
Urea....................	2	92	100	100	+
VP......................	0	0	100		—
Citric acid..............	0	100	100	100	+
MR.....................	100	95	0	100	+
Cellobiose...............	2	95*	99	100	Acid, no gas
H₂S†....................	1	83†	0	0	+
Indol...................	98	6	23	17	—

* Of the 131 "intermediate" strains which attacked cellobiose, 97 produced acid but no gas.

† Vaughn and Levine (1936) medium.

Availability of a compound as a nitrogen source was indicated by production of acid from the glucose in the medium. In all experiments evidence of growth as indicated by increased turbidity, was also recorded.

In table 1 are summarized the results for tests in which the entire collection of cultures was employed.

Adenine was tested with only a few strains (10 *Escherichia*, 10 *Aerobacter* and 8 "intermediates") and xanthine was observed

with 10 *Escherichia*, 10 *Aerobacter* and 50 intermediate strains. The nitrogen of xanthine and adenine was utilized by all the coli strains tested. Since no differential action was evidenced, other cultures were not tested, and these compounds are therefore not listed in table 1.

It was observed that the *Aerobacter* and questionable Voges-Proskauer strains utilized the nitrogen of all the compounds listed; whereas *Escherichia* strains utilized the nitrogen of the pyrimidine uracil only, while the "intermediates" utilized only the nitrogen of urea.

None of the strains of *Aerobacter*, after having acidified the medium, reversed the reaction in nucleic acid, xanthine (questionable reversal for a few strains), or hydantoin media, although they did so in media containing other nitrogen sources. A few *Aerobacter* strains showed beginning reversal of reaction in the uracil medium on the third day, but complete reversion to the original alkalinity did not occur in any case by the fifth day. The "intermediate" and *Aerobacter* strains showed such rapid reversion in the urea medium that observations for short incubation periods (< 24 hours) were necessary in order to detect acid production.

In media containing adenine, xanthine and urea increase in turbidity was well correlated with development of acidity as shown by intensity of color change of indicator. The *Aerobacter* in nucleic acid, and the *Escherichia* strains in uracil, however, produced a distinct acidity without markedly increasing turbidity.

In media employing uric acid or allantoin as sole sources of nitrogen there was perfect agreement between the two indices (turbidity and acidity) for utilization of a test compound when strains of *Aerobacter aerogenes* were used. Of the 39 strains of *Aerobacter cloacae*, however, all produced acid while only 31 per cent (12 strains) showed vigorous growth as evidenced by marked turbidity. In the hydantoin medium, 60 per cent of the *Aerobacter cloacae* strains produced acid after 3 days at 30°C. but in no case was the turbidity greatly increased. Without further chemical study the significance of the differences

observed with the two criteria as indicators of nitrogen utilization is conjectural. Acid production from an available sugar in the presence of a test compound as a sole nitrogen source appeared to have the greater practical value for differentiation of members of the colon group.

Vigor of growth as indicated by increased turbidity was employed as the criterion of availability of carbon, and of both carbon and nitrogen. Observations with 10 *Escherichia,* 8 "intermediates," 5 *A. aerogenes,* and 5 *A. cloacae* strains indicated that nucleic acid, uracil, uric acid, allantoin, hydantoin, and urea were utilized as carbon and as both carbon and nitrogen sources by *A. aerogenes,* but not by the other strains. Adenine sulphate served as an available source of carbon, but not of both carbon and nitrogen, for the *A. aerogenes* strains; it was not utilized by the other strains.

The effect of moderate autoclaving (15 lbs. for 12 minutes, and the medium cooled immediately) on availability of the test compounds as nitrogen sources was observed with nucleic acid, uracil, allantoin, hydantoin, and urea. Inoculation with the above 28 test strains showed that, as nitrogen sources, neither nucleic acid nor uracil was appreciably affected by autoclaving while allantoin, hydantoin, and urea were distinctly modified. Prior to autoclaving, the allantoin and hydantoin compounds had not served as nitrogen sources for either "intermediate" or *Escherichia* strains; after autoclaving they were available as nitrogen sources, for the "intermediate" but not for the *Escherichia* strains. The filtered urea-nitrogen medium supported a vigorous growth and acid production of "intermediates," but *Escherichia* strains did not grow. Autoclaving rendered the medium suitable for *Escherichia* strains. Further work might develop the autoclaved allantoin and hydantoin media as useful in differentiating the "intermediate" from the *Escherichia* strains.

In an experiment employing five *Escherichia* strains it was observed that their differentiation from *Aerobacter* in the allantoin medium was associated with a small inoculum. Heavy seeding from an agar slant presumably introduces some factor which permits abundant multiplication of *Escherichia* strains and therefore invalidates the differential value of the medium.

This necessity for employing a small inoculum is known to hold for several accepted differential tests e.g., Koser's uric acid medium.

DISCUSSION

Since methods for qualitative and quantitative determination of many of the intermediate products of decomposition of nucleic acid are available from the field of physiological chemistry, a study of bacterial fermentation of these compounds should be greatly facilitated. As the *Aerobacter* and questionable Voges-Proskauer strains are able to utilize all of the nitrogen compounds studied it is suggested that the disintegration of nucleic acid by these strains may be analogous to that occurring in the animal digestive tract, and that a study of the bacterial fermentation of these compounds offers a possible method of investigating the course of their digestion in the animal intestinal tract.

Lucke (1931) presented evidence that a great part of the uric acid of the gastric juice and bile is destroyed in the alimentary canal. He found that this destruction commenced in the upper small intestine. Since it is known that strains of *Aerobacter* predominate in that region, the present report makes it logical to assume that nucleic acid or any of its degradation products in the intestine could be readily disintegrated by bacteria as well as by the digestive enzymes of the host.

It would be interesting to know the point in the molecule at which these nitrogen compounds are attacked. The *Aerobacter* strains utilized all compounds tested and therefore offer no clue. That the *Escherichia* and "intermediate" strains could not attack the imidazole ring or that this ring is inhibitory is suggested by their failure to attack uric acid, allantoin, and hydantoin. However, the *Escherichia* strains attacked uracil, and the "intermediates" attacked urea. The evidence for the suggestion offered is slight; other imidazole ring compounds as nitrogen sources must be studied before the suggestion could be accepted as anything other than an hypothesis.

One may then inquire why xanthine and adenine sulphate were utilized as nitrogen sources. At present, there appears to be no logical explanation of the action on xanthine. As to the utiliza-

tion of the nitrogen of adenine sulphate, it is to be borne in mind that various radicals may greatly affect the availability of a compound to bacteria. For instance, glucose and alpha-methyl-glucoside are not attacked with the same ease by the colon group, and methyl urea and thiourea are not equally available nitrogen sources to the same bacteria (de Jong). It may be that the sulphate radical renders the adenine more easily attacked.

Perusal of table 1 shows that, on the basis of utilization of degradation products of yeast nucleic acid in unautoclaved media as sole nitrogen sources, the colon group may be separated into three divisions: one which utilized all of the compounds tested, a second which utilized only uracil, and a third which utilized only urea. The questionable Voges-Proskauer strains were wholly like those of the genus *Aerobacter* with respect to their utilization of these nitrogen compounds and were therefore allocated to that genus.

From table 1 a dichotomous chart may be formed:

Colon Group

VP (+)
yeast nucleic acid (+)
uric acid (+)
allantoin (+)
hydantoin (+)

Aerobacter

VP (−)
yeast nucleic acid (−)
uric acid (−)
allantoin (−)
hydantoin (−)

citrate (+)
uracil (−)
urea (+)
H₂S (+)
indol (−)

Citrobacter

citrate (−)
uracil (+)
urea (−)
H₂S (−)
indol (+)

Escherichia
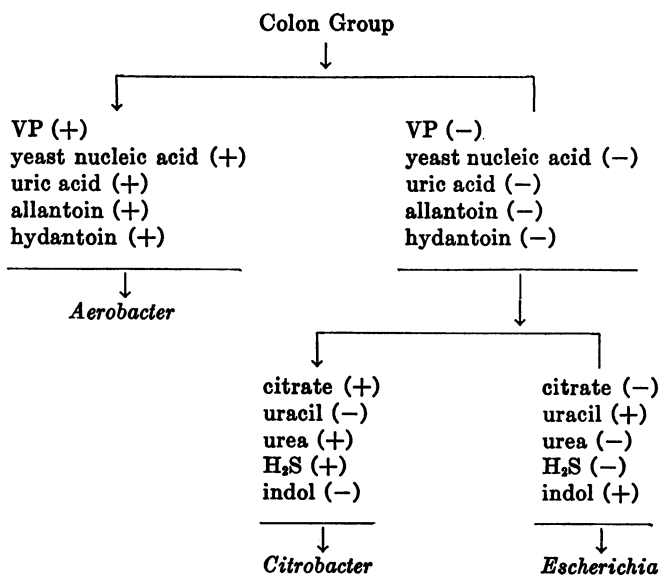
These data may be considered as strengthening the evidence for allocating the "intermediate" strains to a separate genus *Citrobacter*. About 90 per cent of the "intermediates" gave

reactions identical with those of a transfer of the original strain *Citrobacter freundii*, the type species of the genus *Citrobacter*. It is therefore suggested that, if *Citrobacter* is retained, those strains of the coli-aerogenes group having the following characteristics be allocated to that genus:

Gram-negative short rods; do not produce spores; ferment lactose with acid and gas; do not produce acetylmethylcarbinol

DIFFERENTIAL CHARACTERS OF COLON-AEROGENES GROUP

REACTIONS	ESCHERICHIA		"INTERMEDIATE" (CITROBACTER)		AEROBACTER	
	% −	% +	% −	% +	% −	% +
V. P.						
M. R.						
Citric Acid						
Cellobiose						
Indol						
H₂S						
Uric Acid						
Yeast Nucleic Acid						
Allantoin						
Hydantoin						
Uracil						
Urea						

█ Character in which group differs from each of the other groups.
▨ " " " "intermediates" differ from Escherichia
▦ " " " " " " Aerobacter.

FIG. 1

(VP negative); utilize citric acid as a sole source of carbon; generally produce H₂S in appropriate media; utilize urea as a sole source of nitrogen but not yeast nucleic acid, uracil, uric acid, allantoin or hydantoin; and ferment cellobiose with acid, but generally no gas production.

The reactions of the genera *Escherichia*, *Aerobacter* and *Citrobacter* with respect to the twelve characters studied are shown graphically in figure one. It will be noted that the genus *Es-*

cherichia differs from the other two genera in that it does not utilize citric acid or cellobiose as sole sources of carbon, nor urea as a sole source of nitrogen. The genus *Aerobacter* differs from the other two genera in that it is Voges-Proskauer positive, methyl-red negative, and utilizes uric acid, nucleic acid, allantoin and hydantoin as sole sources of nitrogen. The "intermediate" group (*Citrobacter*) differs from both of the other genera in that it produces hydrogen sulphide from appropriate media and, particularly, in that it does not utilize uracil as a source of nitrogen.

The genus *Citrobacter*, as defined above, differs from *Escherichia* in that it is capable of utilizing urea but not uracil as a sole source of nitrogen, is indol-negative, and can utilize citric acid and cellobiose as sole sources of carbon. It differs from the genus *Aerobacter* in that it is Voges-Proskauer negative, methylred positive and cannot utilize uric acid, nucleic acid, allantoin, hydantoin, or uracil as sole sources of nitrogen.

SUMMARY

Studies on the availability of nucleic acid and certain of its degradation products for members of the colon group of bacteria revealed generic differences.

The availability of nucleic acid and its degradation products when correlated with the Voges-Proskauer reaction, citrate utilization, hydrogen sulphide, and indol production, lends support to the allocation of the "intermediate" strains to a separate genus *Citrobacter*.

The colorimetric test based on the production of acid from glucose in a synthetic medium proved to be a convenient and practical method for ascertaining utilization of nucleic acid and its degradation products as sole nitrogen sources.

The nitrogen-availability studies offered a means of classifying strains giving questionable Voges-Proskauer reactions. In the present study six such strains investigated were allocated to the genus *Aerobacter*.

Limited observations indicated that of the colon strains investigated only *Aerobacter aerogenes* utilized nucleic acid, uric

acid, uracil, allantoin, hydantoin, and urea as sole sources of carbon or of both carbon and nitrogen.

Exploratory runs indicated that neither nucleic acid nor uracil was affected as a nitrogen source by moderate autoclaving, whereas allantoin, hydantoin, and urea were distinctly altered. In the present study all media were sterilized by filtration.

A more detailed chemical study is suggested. Investigation of the nitrogen availability of other pyrimidine compounds should prove fruitful.

The genus *Citrobacter* as defined in this paper differs from *Escherichia* in that the former (1) utilizes urea but not uracil as a sole source of nitrogen, (2) utilizes citric acid and cellobiose as sole carbon sources, and (3) produces H_2S but not indol. It differs from *Aerobacter* in that it (1) gives a positive methyl-red test, (2) forms H_2S in proteose peptone ferric citrate agar, (3) does not produce acetylmethylcarbinol (Voges-Proskauer negative), and (4) does not utilize nucleic acid, uric acid, allantoin, hydantoin, or uracil as nitrogen sources.

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